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The Separation of Humic Acids by Chromatography on Controlled-pore Glass

INGVAR LINDQVIST and YLVA LINDQVIST

Department of Chemistry

Abstract. A method for fractionation of soil-extracted humic acid on controlled-pore glass has been investigated. Separations at different pH were performed and the linearity of the separation isotherms was tested by reseparation.

INTRODUCTION

It has been clearly shown that adsorption effects can cause serious problems when separating humic acids by chromatography on Sephadex (Swift & Posner, 1971; Lindqvist, 1967; 1974). By choosing the right conditions these difficulties can be partly or even completely eliminated (Swift & Posner, 1971), but it would be practical to have a chromatographic method with isotherms linear with the concentrations. We therefore started experiments with controlled-pore glass three years ago which proved to be successful for the separation of casein micelles (Almlöf et al., 1977) as well as humid acids. The method was independently tried by Danneberg & Schmidt (1978) with good results. However, we have performed additional studies which we feel worth publishing as a short report.

EXPERIMENTAL

The soil studied was a mud-clay (Kungsängen 1:68, Uppsala) containing 45% (w/w) clay and 10% (w/w) organic material (determined as loss on ignition). The samples were supplied by Agr. B. Weidow, Dept. of Soil Science.

The humic acid was prepared in the following way: After refluxing with benzene, the humic material was extracted with 0.1 m $Na_2P_2O_7$ at pH 7. The humic acid obtained was repeatedly precipitated at pH 1 (HCl), centrifuged and redissolved at pH 7 until no remains could be seen after the redissolution. The pure humic acid was dialysed against distilled water and freeze-dried. The elementary composition was (in weight percentage) C: 50.1, H: 3.2, N: 2.8, O: 33.0 and ash 5.5. The fact that the sum was appreciably lower than 100% (as we had observed several times earlier) was shown to be due to the moisture which was lost before the gas chromatographic analysis (this careful study was made by professor Wolfgang Kirsten and his collaborators). Recalculated to dry weight, the values correspond to C: 53.0, H: 3.4, N: 3.0, O: 34.9 and ash 5.8.

The separation was performed on CPG-10-170B controlled-pore granules having a mean pore diameter of 159 Å (Electro-Nucleonics Inc., Fairfield, N.J., USA) packed in columns 100×1 cm according to the manufacturer's instructions. To achive better resolution, two columns were used in series.

Elution with distilled water made the glass pores inaccessible to humic acid, probably due to electrostatic repulsion. The repulsion was suppressed by elution with buffer solutions containing 0.1 M Na_2HPO_4-NaH_2PO_4 (pH 5.8 and 8.0).

The void volume, V_0, of the columns was determined with Blue Dextran (Pharmacia, Sweden)

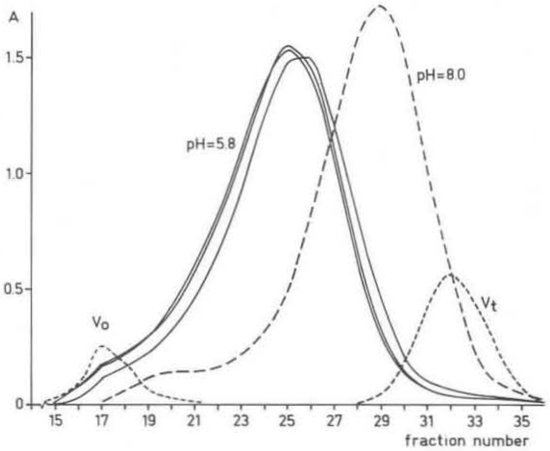

Fig. 1. Chromatogram of humic acids from mud-clay separated on CPG-170 at two different pH values (5.8 and 8.0).

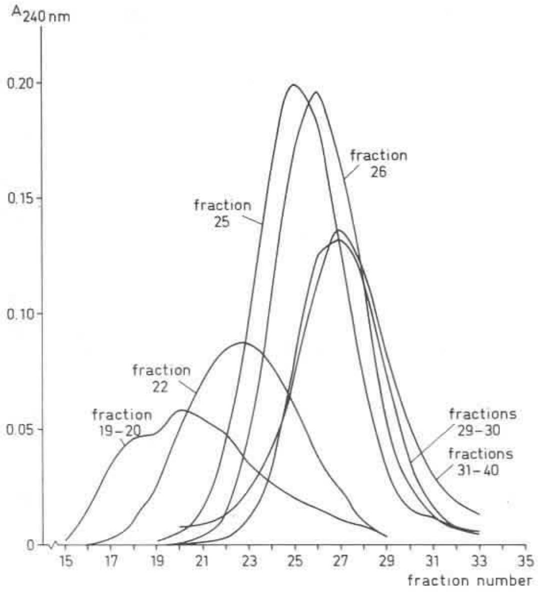

Fig. 2. Reseparation of the fractions on CPG-170 of humic acids at pH 5.8.

and tobacco mosaic virus; the total effective volume with tryptophane.

The humic acid was dissolved and dialysed against the buffer solutions. 250 μl of 1% humic acid was applied and the effluent collected in 3-ml fractions; light absorption was recorded at 240 nm. Neither the speed nor the amount of sample affected the elution pattern. A great number of separations were made.

To prove the linearity of the separation, several corresponding fractions from the different separations of the pH 5.8 preparations were pooled and concentrated by ultrafiltration on Diaflo membranes. These concentrated fractions of arbitrary concentrations were reseparated on the columns.

The collected fractions were characterized by the light absorption spectra between 220 and 700 nm.

RESULTS AND DISCUSSION

The results for three different separations at pH 5.8 and one at pH 8.0 are shown in Fig. 1. The three curves at pH 5.8 give some idea of the maximal variations obtained at different elutions.

The results of the chromatography of a selected number of the collected fractions are shown in Fig. 2. The number of curves represented has been limited for the sake of clarity but these omissions do not influence the discussion. It is quite obvious that the fractions were recovered as would have been expected from the original chromatogram, had a proper separation been obtained for all fractions up to the right side of the large maximum at fractions 25 and 26. The higher fractions came earlier in the re-run, which might indicate that they do not represent a proper separation, but rather a diffusion distribution.

Deviations from linear isotherms have thus not been observed and the separation method seems to be reproducible. It is natural to assume that this is an example of a size separation but it remains to be proved experimentally for humic acid.

If it really is a pure size separation that has been achieved, the increase in pH must have a drastic decreasing effect on the humic acid molecules. As the re-running of the separations does not indicate any association equilibria it is very difficult to explain a size variation with pH. A more penetrating study of the mechanism of the separation on controlled-pore glass is probably necessary.

The E_4/E_6 ($A_{400\ \mathrm{nm}}/A_{600\ \mathrm{nm}}$) for the collected fractions are given in Fig. 3. They indicate the same trend as found by Danneberg & Schmidt (1978): a slightly decrasing value in the first fractions after the void volume and an appreciable increase for the later fractions. When making a comparison with Danneberg & Schmidt's results it must be remembered that we separated the fulvic acids from

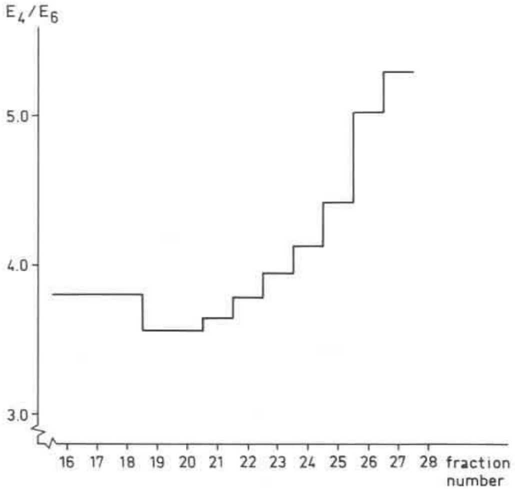

Fig. 3. $A_{400\ \mathrm{nm}}/A_{600\ \mathrm{nm}}$ for humic acids separated on CPG-170 at pH 5.8.

the humic acid before the chromatographic separation. If the separation achieved is due to size differences, it can be concluded that humic acids with very high E_4/E_6 values are low-molecular, while low E_4/E_6 values can be obtained with both high-molecular and low-molecular humic acids.

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Ingvar Lindqvist, Professor
Department of Chemistry
S-750 07 UPPSALA, Sweden

Ylva Lindqvist, Fil. kand.
Department of Chemistry
S-750 07 UPPSALA, Sweden